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VOLTAGE–CURRENT RELATIONS IN NERVE CELL MEMBRANE OF *ONCHIDIUM VERRUCULATUM*

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The voltage clamp technique, introduced by Hodgkin, Huxley & Katz (1952) was first used to analyse the membrane conductance changes during activity in the squid giant axon (Hodgkin & Huxley, 1952*a–d*). Tasaki & Bak (1958), and Dodge & Frankenhaeuser (1958), using a similar technique, examined voltage–current relations in the membrane of a myelinated nerve fibre. However, these investigations were all concerned with nerve fibres.

Recently we have applied a similar technique to nerve cell bodies in the supramedullary ganglion of a puffer fish (Hagiwara & Saito, 1957, 1959). The present work was planned to elucidate further the properties of the nerve cell body membrane by use of the voltage clamp technique. The relation between the membrane potential and membrane current was analysed under normal conditions, as well as in the presence of urethane and tetraethyl ammonium ions (TEA). In this work we used a marine pulmonate mollusc, *Onchidium verruculatum*, which has a number of large nerve cells (100–300μ in diameter) in its central nervous system.

METHODS

The marine pulmonate mollusc *Onchidium verruculatum* is one of the most common species on the Pacific coast of Japan. Some other species, such as *Aplysia punctata* and *Doris japonica*, were examined but *Onchidium verruculatum* was found to be the best material, because the ganglia were only very loosely covered with connective tissue. The sub- and supra-oesophageal ganglia were isolated from the animal together with their nerve bundles. The ganglia were placed horizontally on a glass plate 1 cm in width, and fixed with threads connected to two or three of the nerve bundles. The plate was covered with 2–3 ml. of artificial sea water. The connective tissue strands surrounding the ganglion cells were carefully removed under a binocular microscope. With transmitted illumination the nerve cells in the ganglia were clearly distinguishable from other tissue because of the presence of dense yellow pigment granules inside the cells, and it was usually possible to distinguish more than 10 nerve cells in each preparation.

The micro-electrodes were glass capillary tubes with a tip diameter of less than 0·5μ and filled with 3 M-KCl solution. For some experiments electrodes filled with K_2SO_4 solution were also

employed. The resistances of the electrodes ranged between 10 and 15 MΩ. The indifferent electrode was a silver plate (0·5 × 1·0 cm²) immersed in the solution surrounding the preparation.

The artificial sea water had the following composition (Yamamoto, 1949): (g/l.) NaCl, 27; KCl, 0·7; $CaCl_2.2H_2O$, 1·6; $MgCl_2.6H_2O$, 0·94; $NaHCO_3$, 0·5. The TEA, choline or sucrose sea water was similar to the normal sea water except that the NaCl was replaced by sufficient TEA chloride (Etamon; Parke, Davis and Co.), choline chloride (Merck and Co.), or sucrose to give an isotonic solution. Replacement of Na with TEA or choline to any desired extent was performed by mixing appropriate amounts of the solutions. To change the solution while the electrode was inside the cell, an appropriate amount of one solution was carefully added to the known amount of the solution in which the preparation was immersed. Application of urethane (ethyl-urethane, Merck) was performed similarly with 10% urethane solution, made up in normal or modified sea water.

The pre-amplifier for recording the membrane potential had a low grid current (10^{-11} A) and a variable negative capacity to compensate for the input stray capacity (Haapanen & Ottoson, 1954). The current flowing through the polarizing micro-electrode was recorded as the potential drop across a 5 MΩ resistor inserted between the current electrode and the output of the pulse generator or the output of the feedback amplifier by means of two cathode followers of the same character. Each of the cathode followers had a linear response to a change of input voltage up to ±40 V. The feedback system used for the voltage clamp was similar to that used previously (Hagiwara & Saito, 1959). The amplification of the differential amplifier used for feedback was about 5×10^3. The membrane resistance of the resting cell was 2–5 MΩ and it decreased to 0·1–0·05 of this value when the potential of the interior of the cell was elevated by about 100 mV. Therefore the gain of the amplifier was adequate to maintain the membrane potential at a desired level within an error of 2–4% when the resistance of the current electrode was about 10 MΩ.

All the experiments were carried out at room temperature (20–23° C).

RESULTS

Normal spike potential

The resting potential of cells immersed in normal sea water ranged between 60 and 70 mV. A spike potential appeared when the depolarization produced by an outward current reached 20–30 mV. If a supra-threshold polarizing current was prolonged similar spike potentials appeared repetitively as shown, by records A1 and A2 in Fig. 1. The interval between spikes decreased with increase of current intensity.

The spike had an amplitude of 80–100 mV and was followed by a positive after-potential or undershoot (Fig. 2, A2). At the peak of the undershoot the membrane was hyperpolarized by 10–18 mV. The rising and falling phases of the spike lasted about 2 and 7 msec respectively (at 21° C). The duration of the spike, therefore, was less than 10 msec, and 20–25 msec was necessary for the recovery of the membrane potential from the undershoot.

In a few cases the spike potential produced by an outward current pulse was preceded by a small prepotential (Fig. 1C). Sometimes these potentials failed to grow up to a full-sized spike. Since such abortive prepotentials often appeared on the falling phase of a subthreshold depolarization they presumably represented electrotonic spread to the soma membrane of spikes produced at the axon some distance from the soma. The phenomenon suggests that in such cases the spike is initiated in the axon and conducted back to the soma. This

result indicates that the threshold depolarization at the soma membrane is sometimes somewhat higher than that at the axon membrane.

Stimulation of the roots of the ganglia, especially of those to the pedal muscles, usually produced an antidromic spike in the nerve cell. The antidromic spike was characterized by a step on its rising phase (Fig. 1, B1). This prepotential became clearly visible under anodal polarization of the soma

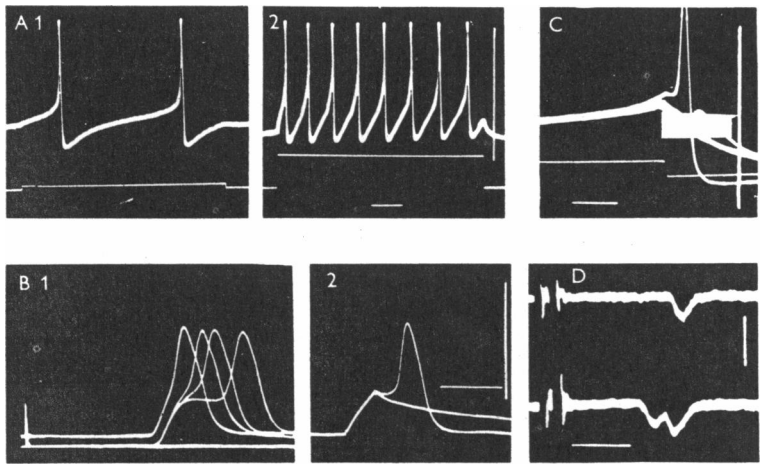

Fig. 1. A1 and A2, spike potential (upper trace) of the normal cell produced by outward current pulses (lower trace) of a long duration. Voltage calibration, 100 mV; time calibration, 50 msec. B, spike potentials produced by antidromic (record 1) and transmembrane stimulation (record 2) of the same cell. Record B1 also shows the effect of anodal polarization of the soma membrane on the antidromic spike. Voltage calibration, 100 mV; time calibration, 20 msec. C, small potentials with and without a full-sized spike. Voltage calibration, 100 mV; time calibration, 20 msec. D, membrane currents produced by stimulation of one axon (upper trace) and two axons (lower trace) of the cell during the voltage clamp of the soma membrane to the resting level; downward deflexion of the trace, inward direction of the membrane current. Current calibration, 0·2 μA; time calibration, 20 msec.

membrane and failed to grow up to a full-sized spike when the potential level at its peak was less than threshold, i.e. the step level. In most cells the step occurred at a level close to that of the critical depolarization found by transmembrane stimulation of the cell soma (Fig. 1, B1 and B2). These results indicate that the prepotential represents an electrotonic spread to the soma membrane of the spike in the axon and that the phenomenon is due to the small safety factor of impulse conduction across the axon-soma junction. Occasionally the step level was substantially below the critical depolarization, and this probably indicates that such a locus occurs across a region of axon some distance from the cell.

One of the remarkable results in the present cell was that the antidromic spike could be produced by stimulating either of two different roots. When a

stimulus was applied to one root during the voltage clamp of the soma membrane at the resting level, a small and rapid inward membrane current was observed at a time corresponding to the rising phase of the prepotential (upper trace of Fig. 1D). Stimulation of the other root also produced an inward current but amplitude and latency were both somewhat different. Simultaneous stimulation of the two roots resulted in a summation of these currents, as is shown by the lower trace of Fig. 1D. These results indicate either that at least two different axons leave from a single cell or that a single axon branches quite close to the cell soma.

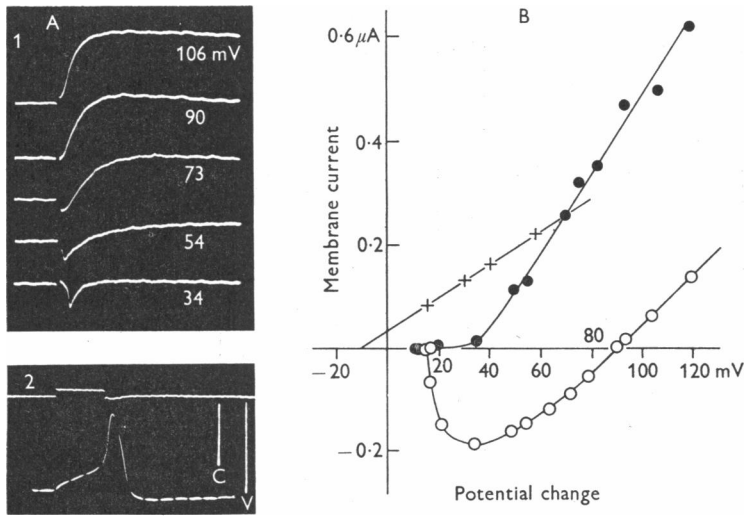

Fig. 2. Current–voltage relations of the normal cell membrane A 1, membrane currents associated with rectangular membrane potential changes. The magnitude of potential change from the resting potential level is listed for each trace; upward deflexion of the trace, outward direction of the membrane current. A 2, spike potential (lower trace) produced by a short outward current pulse (upper trace). Blanking in the potential trace, 5 msec intervals; sweep speed same as in A 1. V, voltage calibration of 100 mV; C, current calibration of 0·5 μA. B, relation between the membrane potential change and the intensity of the membrane current at the peak of the initial surge (○) and at the steady state (●). Outward membrane current, positive on the current axis; depolarization positive on the potential axis, taking the resting potential level as zero. Crosses show the voltage–current relation obtained by the two-step voltage clamp experiment at the steady state.

Current–voltage relation of the normal cell membrane

A series of records in Fig. 2, A 1 shows the membrane currents when the membrane potential of the cell was clamped at various levels. In these records an upward deflexion corresponds to an outward current through the cell membrane. The large capacitative current that flowed at the onset of a sudden potential change is discernible only as a short break in the records. After the capacitative current was over, an initial inward surge appeared, followed by a

gradual change to an outward current. The current intensities at the peak of initial surge (open circles) and at the final steady level (filled-in circles) are plotted against the changes in the membrane potential in Fig. 2B. In this figure, outward current is positive on the current axis, depolarization is positive on the potential axis, and resting potential corresponds to zero change of membrane potential. The potential at which the membrane current at the moment corresponding to the peak of the initial surge became reversed was

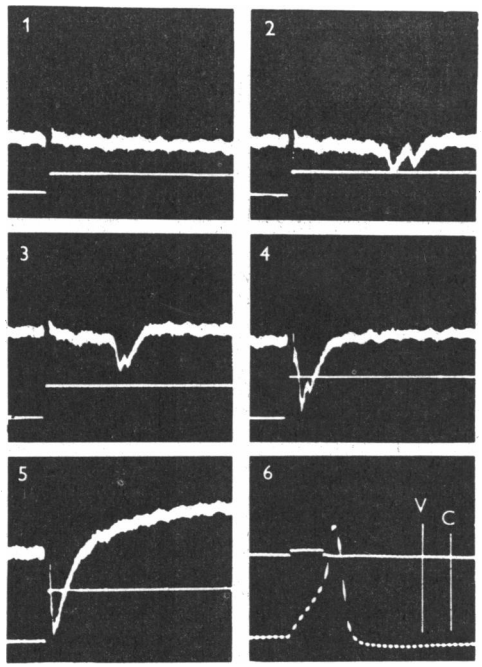

Fig. 3. Membrane currents (upper trace) associated with smaller rectangular depolarizations (lower traces). Blanking in the potential trace in record 6, 2 msec; V, voltage calibration of 100 mV; C, current calibration of 0·2 μA.

80–100 mV more positive (inside relative to outside) than the resting potential level. This value coincided with the membrane potential at the peak of the spike recorded from the same cell. The current–voltage relation approximated to a straight line around this potential level. The membrane resistance estimated from the slope of this straight line was 1/8–1/20 of the resting resistance of the same cell membrane.

Records in Fig. 3 were obtained with higher sensitivity of current recording in order to show membrane currents during voltage clamp of the cell membrane to relatively small positive levels. When the potential change reached a certain value, which was usually similar to the critical depolarization obtained by an outward current pulse, a small inward surge appeared with a

considerable latency. Often the inward surge at this potential showed double peaks, or was composed of two separate small surges. Increasing the clamped membrane potential decreased the latency and at the same time these component surges became synchronized. The synchronization caused a summation of surges, and therefore resulted in an increase of intensity at the peak. These surges were very similar to those obtained by antidromic stimulation. If these inward surges are contaminated by the current due to spikes at the axon somewhere beyond the space clamp region, they do not immediately indicate a sign of activity at the cell soma.

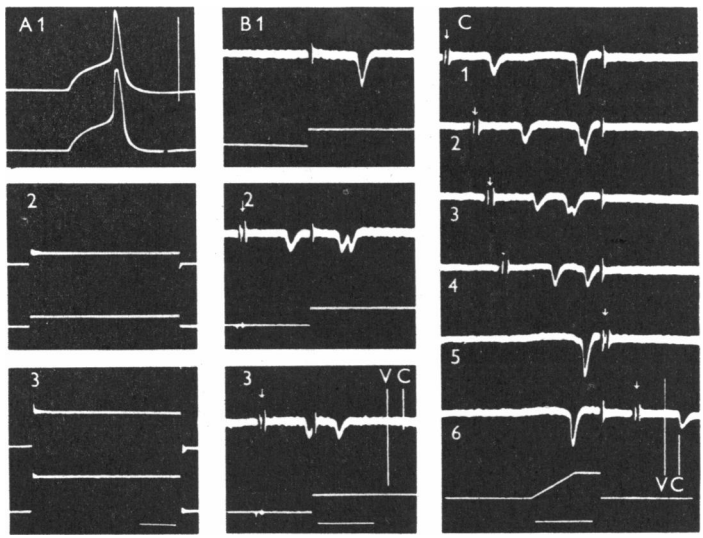

Fig. 4. A, potential changes simultaneously recorded through two internal micro-electrodes inserted into the same cell when a threshold outward current pulse was applied through a third internal electrode (record 1), and when the membrane potential was clamped with this electrode and one of the recording electrodes (records 2 and 3). Upper trace in each record was obtained through the recording electrode used for the voltage clamp. Voltage calibration, 100 mV; time calibration, 50 msec. B and C, effect of antidromic stimulation on the initial inward surge associated with a small depolarization; arrows in records indicate moments of antidromic stimulation. In B and C, V, voltage calibration of 100 mV; C, current calibration of 0·2 μA; time, 50 msec.

In order to obtain the current–voltage relation of the actual cell membrane by the present technique it is necessary that the whole of the membrane of the cell should be under the space clamp and also that the current flowing through the membrane of axon or dendrite should be negligible. For examining the first point the membrane potential change was recorded through a third micro-electrode inserted into various regions of the cell membrane while the voltage clamp was carried out with two other internal electrodes, and was compared with the potential recorded through the electrode used for feedback.

As is shown by Fig. 4 A, they were always in good agreement. Therefore the present experimental conditions satisfy the first point.

Since the present nerve cell has an axon or axons, some fraction of membrane current should flow through the membrane of the axon. When a constant inward current of a relatively small intensity was applied to the cell membrane it hyperpolarized the membrane with a time course that was close to exponential. This provides some evidence that the current flowing through the axon membrane is small compared with that through the soma membrane. However, this conclusion holds only for the resting condition, or at least when the membrane properties are practically uniform between the cell and the axon. When the clamped membrane potential in the cell itself is at a level slightly, say 25 mV, less negative than the resting potential, the membrane of the axon some distance from the cell depolarizes with a time course determined by the time constant of the axon membrane and the internal resistance between this region and the cell soma. If the distance between these two regions is not very large compared with the space constant of the axon, the final level of the potential change at this axon membrane should not be much smaller than that in the soma, and therefore it may be enough to produce a spike potential because the membrane of this region may not be well clamped. Such a spike potential should result in a small inward surge in the recorded membrane current. On the other hand, the initial inward surge due to the cell membrane itself may not be easily recognizable, since the potential change applied by the voltage clamp is not large. In order to examine the above possibility, the interaction was observed between the inward surge due to antidromic stimulation and that produced by applying a small depolarization with the voltage clamp. In Fig. 4 B, record 1 shows an inward surge associated with a small rectangular depolarization. When the antidromic impulse preceded this surge by a certain interval the latter separated into two surges (record 2). Decrease of the interval caused the second component of these two to disappear. The result indicates that the directly produced surge is composed of at least two components, one of which is probably due to excitation of the region at which the antidromic impulse terminates. Since the antidromic surge was produced under the voltage clamp of the soma membrane to the resting potential level the antidromic spike terminated in the axon some distance from the cell. The separation of the double peaks of the initial surge associated with a small depolarization may be due to asynchronous spike initiation at two axons for a single nerve cell. Figure 4 C shows a similar series of records obtained with a linearly increasing voltage pulse. As is shown by traces 5 and 6, the directly produced surge also left a refractory period for the antidromic surge. The least interval between the directly produced and antidromic surges was twice the conduction time through the antidromic pathway, the latter being measured as the interval between the antidromic stimulation and surge. The

phenomenon indicates that a spike probably originates at the axonal region and travels distally along the axon, and therefore collides with the antidromic impulse.

The foregoing results show that the initial surge associated with a small depolarization of the membrane is greatly contaminated by current due to the spike originating at the axon. The effect of a preceding antidromic surge was also observed on the initial surge associated with a larger positive-going potential change. It was usually found that the amplitude of the initial surge was not decreased significantly by such an antidromic surge when the potential change was larger than about 50 mV. Therefore, the results on the initial surge obtained for higher positive-going potential changes give the current–voltage relation of the membrane under the space clamp with reasonable reliability. Even though the membrane under the space clamp includes the soma membrane it may also include the membrane of the initial short length of the axon. In such a case the eight- to twentyfold increase of the membrane conductance associated with the initial surge is some sort of average value for these membranes.

Since the final steady state of voltage clamp is not associated with an action potential of the unclamped axon membrane, the current–voltage relation obtained under the present experimental condition can be compared with those obtained under the resting condition. As is shown in Fig. 2B, the intensity of the membrane current was similar to that expected from the resting membrane resistance for membrane potential changes smaller than about 30 mV. However, the slope of the current–voltage relation became much steeper for larger membrane potential changes. Although the intensity of current varied approximately linearly with the membrane potential change, the straight line did not actually cross the potential axis. Furthermore, it was obtained a certain period after the membrane potential was changed, and therefore the equilibrium potential across the membrane in the absence of applied current might have changed during this period. For these reasons the two-step voltage clamp technique was employed to estimate the equilibrium potential and conductance of the membrane during the final steady state. After the membrane current reached a steady level the membrane potential was suddenly shifted to a second level. Subtracting an appropriate capacitative current from the membrane current, the current just following the sudden potential change was obtained and was plotted against the membrane potential of the second level as shown by the crosses in Fig. 2B. The points fit a single straight line. If the conductance and equilibrium potential are not changed rapidly with the potential shift, the slope of the straight line should indicate the membrane conductance at the steady state and the equilibrium potential for the corresponding state should be given by the intersect on the potential axis. The apparent equilibrium potential was slightly more negative than the resting

potential level. Almost the same value of equilibrium potential was obtained with any other large potential change during the first step. The conductance of the membrane thus measured at the steady state was smaller than that given by the slope of the straight line relationship between the membrane potential and the final steady current and increased with increasing change of the membrane potential. At 100 mV depolarization, for instance, the conductance at the steady state was 10–20 times as large as the resting conductance of the same membrane. This change in the membrane conductance corresponds to the so-called 'delayed rectification' observed for the squid giant axon (Cole, 1941; Hodgkin & Huxley, 1952*d*).

The foregoing results suggest that the membrane current obtained by voltage clamp technique is apparently composed of two components, as was found in the squid giant axon by Hodgkin, Huxley & Katz (1952), and by Hodgkin & Huxley (1952*a*, *b*, *c*, *d*). They separated these components by removing the sodium ions in the external solution. Similar experiments were also undertaken in the present nerve cells by applying urethane and tetraethyl ammonium in the external medium.

Effect of urethane

In the presence of 2% urethane the amplitude of the spike potential decreased, and finally the cell became incapable of producing an all-or-none type of spike; but no appreciable change appeared in the resting potential. After the decrease of spike potential had reached a steady state, the membrane currents were recorded during a voltage clamp (Fig. 5, A2). Comparing these currents with those obtained from the same cell before applying urethane (Fig. 5, A1), it was found that the initial surge was substantially changed by urethane. In the present example the effective equilibrium potential for the initial surge decreased from its normal value of 80 mV to 60 mV in the presence of urethane (Fig. 5C). The effective conductance of the membrane for the initial surge, obtained from the slope of current–voltage relationship at 60 mV, was much smaller than the conductance of the same membrane before application of urethane (Fig. 5C). These changes evidently corresponded to a decrease of the spike amplitude. In contrast to this no appreciable change was observed in the final steady current. There are marked similarities between the membrane current produced by the presence of urethane in the present nerve cell and that of a squid giant axon observed in the absence of external sodium (Hodgkin & Huxley, 1952*a*).

To examine the effect of sodium lack in the present cell the behaviour of the cell membrane was observed in choline sea water or sucrose sea water. The spike height showed a great decrease after immersion for 60 min or longer in these solutions. The decrease of the spike height always accompanied a corresponding decrease of initial surge obtained by voltage clamp technique, while no clear change was observed in the rectification of the membrane. Recovery

was usually observed when the external choline or sucrose solution was replaced by normal sea water. The behaviour of the present nerve cell in the sodium-free solution is, therefore, similar to that of the squid giant axon.

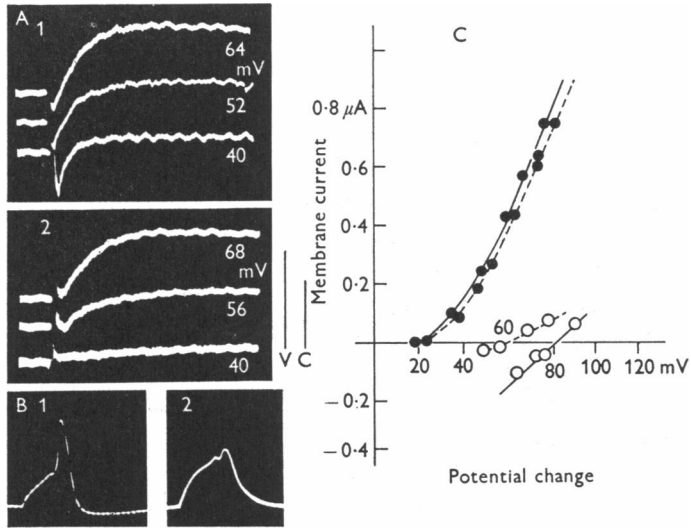

Fig. 5. Effect of urethane 2% on the cell membrane. Membrane currents associated with rectangular membrane potential changes in the normal cell are shown in A 1, and those obtained after the application of urethane in A 2; the magnitude of the membrane potential change from the resting level is indicated for each trace. Records B 1 and B 2 show the spike potentials corresponding to records A 1 and A 2 respectively. V, voltage calibration of 100 mV; C, current calibration of 0·5 μA; blanking in record B 1, 2 msec, is common for all the records. C, relation between the membrane potential change and the intensity of the membrane current at the peak of the initial surge (○) and at the steady state (●). The continuous curves indicate the relations obtained before, and the interrupted curves the relations obtained after the application of urethane.

Effect of TEA

When TEA was applied to the cell the duration of the spike potential was markedly increased. The record in Fig. 6 A was taken with continuously moving film while the cell was stimulated by short outward current pulses at a frequency of one per second. The bar in the record indicates the period during which 0·5 ml. of TEA sea water was added to 2·0 ml. of normal sea water surrounding the preparation. In the final state, therefore, one-fifth of the sodium in the medium was replaced by TEA. A few seconds after the start of application of the solution the spike potential began to be prolonged. In 30 sec or so the prolongation reached a steady state which depended on the final concentration of TEA. Occasional peaks on the spikes in the record are due to the superposition of the applied pulses on the spikes. At low concentrations of TEA the change was a small decrease in the rate of fall of the spike potential. As the concentration increased the prolongation became more

marked. Replacement of one-sixth of the sodium by TEA produced the maximum prolongation. At room temperature (21° C) the maximum duration of the spike was 50–60 msec while that of the normal spike was less than 10 msec. TEA did not change the resting potential of the cell. A small reduction of the spike height was usually observed and this depended on the final concentrations of TEA and Na in the external medium.

Besides a marked increase of the spike duration, TEA caused a typical change in the shape of the spike. The membrane potential fell at an extremely slow rate immediately after the peak and thus formed a plateau. The plateau

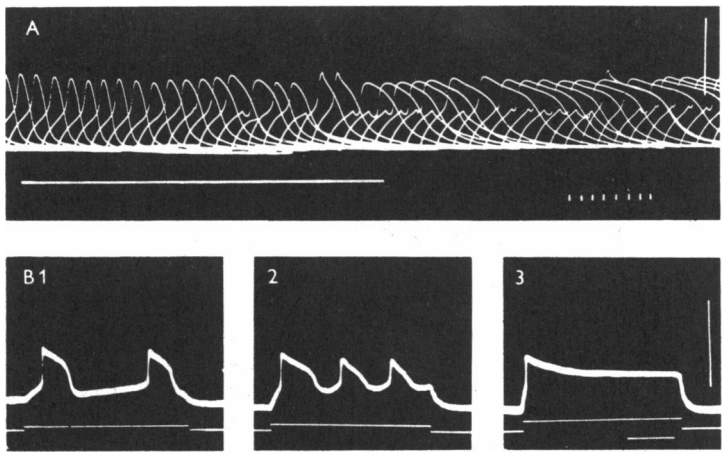

Fig. 6. A, spike potentials produced by a succession of short outward current pulses at 1/sec during the addition of TEA sea water (0·5 ml.) to the external normal sea water (2·0 ml.). The bar indicates the period of mixing the solutions. Initial rapid peaks seen in some of prolonged spikes were due to the applied current pulse which flowed in the early phase of the spike. Voltage calibration, 100 mV; time calibration for each trace, 5 msec. B, spike potentials (upper trace) of the TEA-treated cell produced by relatively long outward current pulses (lower trace). Voltage calibration, 100 mV; time calibration, 100 msec.

was terminated abruptly by a shoulder which was followed by a slight undershoot. In other words, the spike potential became very similar to that of cardiac muscle (Weidmann, 1956). At low concentrations of TEA the plateau was sometimes preceded by an initial rapid fall from the peak just as in the spike potential of the mammalian Purkinje fibre (Draper & Weidmann, 1951). However, in such cases the spike duration was usually not very long. Similar prolongation of the spike potential by TEA has also been reported for vertebrate myelinated nerve fibres (Lorente de Nó, 1949), for crustacean muscle fibres (Fatt & Katz, 1953), vertebrate muscle fibres (Hagiwara & Watanabe, 1955) and for the squid giant axon when TEA is injected internally (Tasaki & Hagiwara, 1957).

Prolonged steady currents in the outward direction produced repetitive

spikes in TEA just as in normal solution (records in Fig. 6B). However, at a certain intensity the fall of the first spike potential from its shoulder became incomplete and this was followed by spike potentials of smaller duration and amplitude (Fig. 6, B2). With higher current intensities the plateau of the first spike continued as long as the current lasted (Fig. 6, B3).

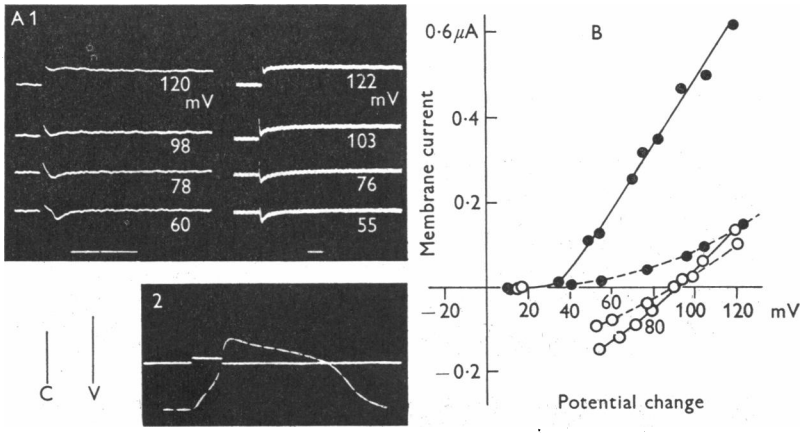

Fig. 7. Current–voltage relations of the TEA-treated cell membrane; 1/5 of the Na in the external medium was replaced by TEA. A1, membrane currents associated with rectangular membrane potential changes; the magnitude of the potential change from the resting level is indicated for each trace; right and left columns were obtained with different sweep speeds. Time calibration, 25 msec for both. A2, spike potential (lower trace) produced by a short outward current pulse (upper trace); blanking in the potential trace, 5 msec. Records 1 and 2 were obtained from the same cell. V, voltage calibration of 100 mV; C, current calibration of 0·5 μA. Records in Fig. 2A and in Fig. 6A were obtained with the same cell before and after applying TEA. B, relation between the membrane potential change and the intensity of the membrane current at the peak of the initial surge (○) and at the steady state (●). The continuous curves indicate the relations obtained before, and the broken curves those obtained after the application of TEA.

The current–voltage relation obtained by the voltage clamp technique was strongly modified by the application of TEA. Figure 7A, 1 shows records taken after replacement of one-fifth of Na in the external sea water by an equivalent amount of TEA. Records taken before the addition of TEA to this cell are shown in Fig. 2A, 1. The current–voltage relations obtained before and after TEA treatment are plotted in Fig. 7B. A small decrease of initial surge was observed in the presence of TEA. In contrast to this the current at the steady state was greatly decreased by TEA. The conductance and equilibrium potential of the membrane in the final state were examined by two-step voltage clamp technique and it was found that the smaller outward current at the steady state was not due to a change of equilibrium potential but to a smaller increase of the membrane conductance, even though the latter was larger than the resting membrane conductance. Although rectification showed a marked

decrease, no significant change was observed in the resting membrane resistance in the presence of TEA.

The initial surge in a TEA-treated cell disappeared after addition of urethane while the final steady current stayed unchanged (Fig. 8B). In a few experiments TEA solution was applied to the nerve cell which had previously been narcotized by urethane. TEA diminished rectifying action of the membrane in the narcotized cell just as in the normal cell. These results show that the

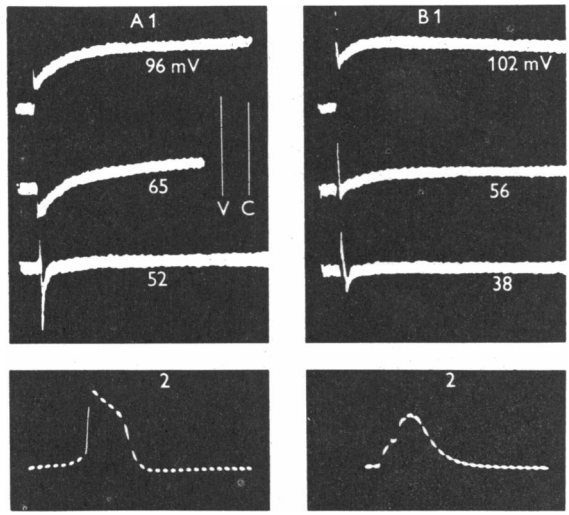

Fig. 8. Effect of urethane (2%) on the TEA-treated cell membrane; 1/4 of the Na in the medium was replaced by TEA. Membrane currents associated with rectangular membrane potential changes in the TEA-treated cell before (A1) and after (B1) the application of urethane. Records A2 and B2 show the corresponding spikes. Blanking in the spike recordings, 20 msec; V, voltage calibration of 100 mV; C, current calibration of 0·2 μA.

effect of TEA is mainly on the rectifying action. However, it is worth while to note here that a small but significant rectification is always present in the TEA-treated cell, even though it is much less obvious than in the normal cell.

Abolition of prolonged spike potential

When a weak pulse of inward current was passed through the membrane during the plateau phase of the spike potential of the TEA-treated cell, the membrane potential went down during the period of current flow. After termination of the pulse, the potential returned to the level which would have been attained had the pulse not been applied. When the pulse intensity exceeded a certain value, however, the membrane potential did not rise after the end of the pulse but went down to a level which was independent of the applied current. In other words, abolition of the rest of the spike potential occurred (Fig. 9B). When the current intensity was adjusted to threshold for abolition,

the membrane potential stayed at a constant level for a while and then went either up or down, as is shown by record B 1 of Fig. 9. The threshold membrane potential for abolition rose continuously during the course of the spike potential. Although the abolition of the spike potential in the present nerve cell was 'all-or-none', the rate of potential change with which the membrane potential went either up or down depended on the intensity of current. Record B 1 of Fig. 9 was taken by using current pulses of two different intensities, the one being just threshold intensity for abolition and the other

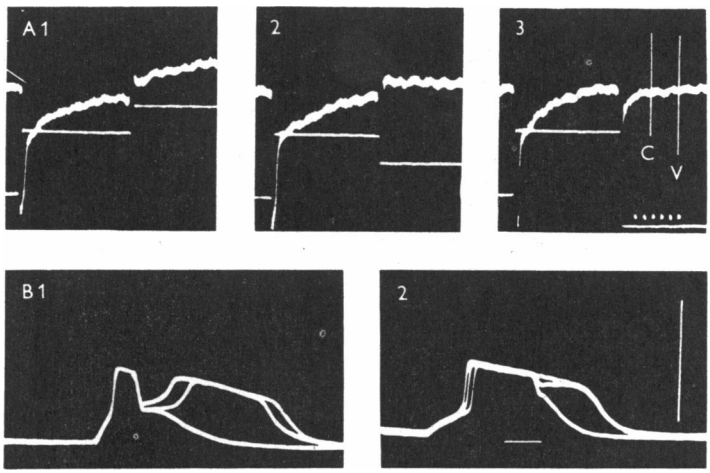

Fig. 9. A, membrane currents of the TEA-treated cell obtained by the two-step voltage clamp technique. The upper trace shows the membrane current and the lower the membrane potential change. V, voltage calibration of 100 mV; C, current calibration of 0·2 μA; time, 5 msec. Records in B show abolition of the prolonged spikes of TEA-treated cells produced by short inward current pulses applied at the plateau phase of the potential. Voltage calibration, 100 mV; time calibration, 20 msec.

subthreshold. The membrane potential recovered at a faster rate in the latter case. In the experiments shown in Fig. 9A the membrane potential was shifted to various new levels at the moment when the membrane current reached zero during the first step of the voltage clamp. The potential level of the first step was about 50 mV. When the membrane potential of the second step was in a certain range, decrease of the potential inside the cell was associated with development of outward current and this corresponded to a negative membrane resistance. This negative value of resistance indicates that the membrane potential would go down automatically if the current supplied from the feedback system was terminated after the start of the second step of voltage clamp; in other words, abolition should occur. A larger negative resistance associated with the second step corresponded to a larger rate of fall in the abolition. The results are very similar to those already observed in

cardiac muscle (Weidmann, 1951, 1956), myelinated nerve fibre (Tasaki, 1956) and in the squid giant axon injected with TEA solution (Tasaki & Hagiwara, 1957).

Membrane change associated with hyperpolarization

An inward current pulse hyperpolarized the cell membrane. The hyperpolarization developed with an approximately exponential time course and the final steady level depended on current intensity (Fig. 10, A1). When the current intensity was not very high, the current–voltage relation showed that the membrane resistance was constant, being about 2·5–4·5 kΩ. cm². However,

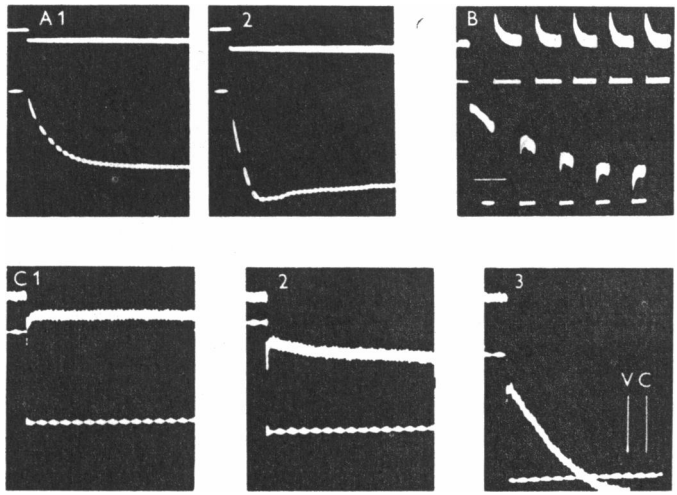

Fig. 10. Current–voltage relations obtained with a hyperpolarized cell membrane. A, potential changes (lower trace) associated with rectangular inward current pulses (upper trace). B and C, membrane current (upper trace) associated with rectangular hyperpolarizations under a voltage clamp (lower trace). V, voltage calibration of 100 mV for all the records; C, current calibration of 0·5 μA for all the records; blanking in the potential trace, 20 msec and time calibration in B, 100 msec.

for higher current intensities the potential change decreased after reaching a peak and attained a final potential level which was smaller than that expected from the resting membrane resistance (Fig. 10A2).

If the membrane potential was clamped to various large negative levels, the inward current increased enormously during the voltage clamp (Fig. 10C). The current increased at a relatively slow rate and the effect remained for a long time after the membrane potential returned to resting level. This was examined by a second rectangular hyperpolarization at various times after the termination of the first potential change (not shown in the figure). If a succession of potential changes of short duration was applied, the current intensity increased in the same way as if a single long hyperpolarization had been applied

(Fig. 9B). This also indicates that the effect lasted for a time after the potential change. Although the effect remained for as long as 1–3 sec after recovery of the membrane potential, the change of the membrane was completely reversible.

To determine whether the large hyperpolarization set up an elevation of the membrane equilibrium potential or an increase of the membrane conductance the two-step voltage clamp technique was employed (Fig. 11B). The thicker curve in Fig. 11A gives the current–voltage relation at the steady state. The points A, B, C,... were obtained from the two-step voltage clamp technique. These points fit a straight line and the membrane conductance determined by

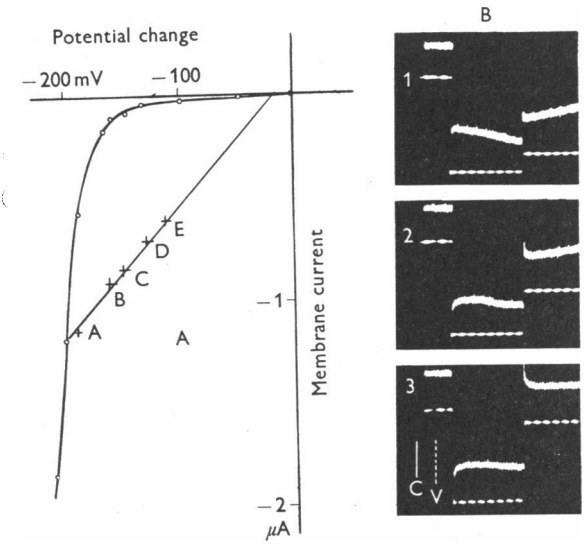

Fig. 11. A, relation between the membrane current and the magnitude of hyperpolarization obtained at the steady stage (thicker curve); inward membrane current or hyperpolarization is negative on each axis. Records in B were obtained by the two-step voltage clamp technique; upper trace and the lower trace show the membrane current and the membrane potential change respectively. V, voltage calibration of 100 mV; C, current calibration of 0·5 μA; blanking in the potential trace, 20 msec.

the slope was about 30 times the resting conductance. The membrane equilibrium potential estimated from the intersect on the potential axis was slightly but significantly more negative than the resting potential. Since this change of the equilibrium potential tends to decrease the membrane current during hyperpolarization the increase of current found with hyperpolarization is due to an increase of membrane conductance.

This increase of membrane conductance during hyperpolarization was not affected by application of urethane or TEA. Furthermore, the phenomenon does not seem to be due to any specific effect of chloride ions which might flow into the interior of the cell from the KCl-filled micro-electrode during the

application of the inward current, since there was no appreciable difference, either in the conductance or in the effective equilibrium potential, when a K_2SO_4-filled electrode was used.

DISCUSSION

The voltage–current relations obtained with the present nerve cell immersed in normal sea water are similar to those obtained with the puffer nerve cell (Hagiwara & Saito, 1957, 1959), myelinated nerve fibre (Tasaki & Bak, 1958; Dodge & Frankenhaeuser, 1958), and squid giant axon (Hodgkin *et al.* 1952; Hodgkin & Huxley, 1952*a*, *b*, *c*, *d*) in the following points. The membrane current associated with a relatively large rectangular depolarization is characterized by two component currents. The initial transient surge correlates with the amplitude of the spike potential obtained in the absence of a voltage clamp. There follows an outward current which develops to a steady level with a certain delay and lasts as long as the membrane potential stays at a high depolarization level. The latter seems to be similar to the current associated with the delayed rectification in the squid giant axon (Cole, 1941; Hodgkin & Huxley, 1952*d*).

As has been described above, the initial surge showed a marked decrease or even disappeared in the presence of urethane or in the absence of sodium ions in the external solution. However, urethane as well as sodium lack did not affect the rectifying action which was measured by the voltage–current relation at the steady state of voltage clamp. Very similar changes were observed in the puffer nerve cell (Hagiwara & Saito, 1959) when the preparation was in poor condition, for example, with poor oxygen supply. Similar changes have been obtained with a squid giant axon immersed in a solution containing reduced sodium (Hodgkin & Huxley, 1952*b*) and in the frog myelinated nerve fibre in the absence of external sodium, under narcosis as well as when refractory (Tasaki & Bak, 1958). Under these conditions there is commonly some decrease in the effective equilibrium potential and in the slope of the current–voltage relation at the peak of the initial surge. These changes in the initial surge correspond to the decrease of amplitude of the spike potential in the absence of a clamp.

Although TEA does not change the resting membrane resistance it greatly diminishes the rectification of the cell membrane while the initial surge is not much changed. There is some indirect evidence for a similar decrease in rectification in the striated muscle fibre with TEA (Hagiwara & Watanabe, 1955). In the squid giant axon externally applied TEA acted similarly to choline, which is considered to be inert. However when injected internally TEA shows a definite tendency to decrease rectification (Tasaki & Hagiwara, 1957). The decrease of rectification is generally associated with the prolonged fall of the action potential obtained in the absence of a voltage clamp.

For a hyperpolarization of less than 150 mV there was no change in the membrane resistance, but when the amount of hyperpolarization exceeded this value a gradual decrease of membrane resistance occurred. A similar phenomenon has been reported for *Aplysia* nerve cells by Tauc (1955) and also for spinal ganglion cells by Ito (1958). According to Hodgkin (1947) a similar decrease of hyperpolarization occurs at the membrane of the *Carcinus* nerve fibre during a strong inward direct current, and termination of the current is followed by a depolarization which is often associated with the so called 'Ritter's tetanus'. From these results he suggested that an increase of simple leakage occurred at the membrane, probably due to some sort of break-down of the membrane by the strong inward current. However, in the present case although the conductance increases no such depolarization follows the termination of current, presumably because the equilibrium potential is in the direction of a slight hyperpolarization. The change of equilibrium potential does not seem to be due to a specific effect of chloride ions which flow into the interior of the cell from the current micro-electrode. The insensitivity of the phenomenon to urethane indicates that it has no direct relation to the mechanism of excitation. Since TEA does not suppress the conductance increase of this type, the mechanism of the increase seems to be different from that of the one associated with the depolarization.

SUMMARY

1. Investigations were made on the nerve cell membrane of a pulmonate mollusc, *Onchidium verruculatum*. Two micro-electrodes were inserted into the same cell and the membrane properties were examined under various experimental conditions while the membrane potential or current were controlled.

2. The resting potential of the cell was 60–70 mV and depolarization beyond a certain value (20–30 mV) produced a spike potential with a peak voltage of 80–100 mV which was followed by a positive after-potential.

3. The membrane current of the clamped normal cell associated with a rectangular depolarization was characterized by a rapid initial inward surge followed by a slowly developing outward current. The former was associated with a conductance increase of the membrane to an equilibrium potential which was 80–100 mV above the resting potential level, and the latter was associated with a conductance increase to an equilibrium potential which corresponded to a small hyperpolarization level.

4. External application of urethane decreased the amplitude of spike potential as well as the initial surge, while the final outward current was not affected. Similar results were obtained in choline or sucrose sea water.

5. External application of TEA increased the duration of spike potential. The characteristic change obtained by the voltage clamp technique was marked decrease of final steady outward current.

6. Brief inward pulses of sufficient size applied during the plateau of the TEA potential abolished the rest of the spike.

7. An enormous increase of membrane conductance is associated with a hyperpolarization larger than about 150 mV.

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